MICROBIOLOGICAL METHODS IN BIOCHEMICAL INVESTIGATIONS

Translation No. 632

RT-2693

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Uspekhy Sovrem Biol. (Progress in Modern Biology),
Vol XXXIII, No 3, 1952, Moscow-Leningrad, pages 365-379 V. S. Asatiani

The problems of the utilization of microorganisms for quantitative biochemical analysis have been briefly covered in the review paper by Paley (1940), Rabotnova (1947), Iyerusalimskiy (1947), Odintsova (1949), and Mardashev (1950).

Our paper presents a more complete review of the methods for determining aminoacids. It was prepared in a somewhat different format and takes into account some of the most recent literature data on this problem.

One of the bases for the development of methods for the use of microbes as test reagents for various organic (and inorganic) compounds encountered in animal organisms are the investigations by Nentskiy (1881) and other of the chemical composition of microbes and by Ushinskiy (1893) who showed that the culture of microbes in synthetic media is possible. Later, the talented Russian scientists Vinogradskiy (1897) and, particularly, Omelyanskiy pointed out that it was possible to use microbes as chemical reagents. One of the papers by Omelyanskiy (1924) was actually titled "Microorganisms as Chemical Reagents". Still later, the contribution by Soviet scientists toward the development of microbiological methods did much to adapt these methods for biochemical investigations.

During the last decade, microbiological methods for determining aminoacids, vitamins, and other compounds encountered in organisms came into wide use because of their simplicity and universality (simple equipment, technique of determination, and standard reagents for all determinations). A good thermostat or a thermostat room, autoclave; burets and microburets, nephelometer, analytical balance, ordinary laboratory were (including a sufficient number of standard-type test tubes), and reagents is essentially all that is required to perform a microbiological quantitative analysis. All this is available to any laboratory. In addition, it is possible, in most cases, to dispense with the nephelometer.

A shortcoming of this method is the requirement of presently still critical reagents for the synthetic media which are usually of complex composition. As we shall see below, our own scientists were, in various instances, successful in overcoming this basic difficulty by developing methods which utilize media of more simple composition. The laboriousness of the method is another of its shortcomings. In addition, for an accuracy of the order of 5-10 percent, which is quite satisfactory for ordinary biochemical analyses, and for a high sensitivity, which would permit the analysis of very small samples (for the determination of aminoacid composition, e.g., 20-30 mg. of protein are required), the microbiological method cannot yet compete, in this respect, with several other methods of biochemical analysis having an accuracy of up to 1 percent (and sometimes even fractions of a percent).

However, the sensitivity, the simplicity of the procedures, and their uniformity are factors which rather favor the use of microbiological methods in biochemical investigations.

The specificity of microbiological methods still has certain limitations. Thus, for example, it was shown by Meysel (1950) that, on the basis of thiamin (vitamin B_1), all microbes can be classified into five groups, of which some exhibit a growth reaction for the pyrimidine or thiazole portion of the molecule of vitamin B_1 or for both portions of the molecule, but are not linked in the same manner as in the thiamine molecule. It is well known that the vitamin activity of these related compounds, with respect to higher animals and humans, is not the same; this should be taken into account in order to avoid erroneous results.

Shultz and others (1942) proposed to eliminate a possible source of error by conducting a parallel, blank sulfite experiment. In this manner, by eliminating the influence of the thiamine alone on the growth reaction, it is possible to obtain, by difference, the figure corresponding to the content of the actual vitamin B_1.

Some analogs of riboflavin can be used as substitutes for it, in determining the influence on the growth reaction of lactobacilli (Lamboov, 1951).

Tryptophane, anthranilic acid, and possible "predecessors" of nicotinic acid can be used as a substitute for this vitamin in feeding certain microbes (Davis and others, 1951).

Some strains of bacteria can synthesize biotin on the basis of pimelic acid; this should be taken into account when they are utilized for the microbiological determination of biotin (Du Vigneaud and other, 1942; Melville, 1944). According to Meysel (1950), some microorganisms require a complete molecule of pantothenic acid, while other are content with beta-alanine. In feeding Streptococcus faecalis, pyridoxine (and probably pyridoxamine) can be replaced with alanine, provided the latter is furnished in considerable amounts (Snell and Guirard, 1943; see also Holden and other, 1949). According to Kicher (1949) and Kiditschek and other (1949) reducing substances can replace vitamin B_{12} in feeding lactobacilli. According to Koft and others (1950), the decomposition products of folic acid produce a more active growth reaction of microbes than pure folic acid. These data, however, require verification.

It was established by Meysel (1947) that, in the case of many microbes, portions of the cleaved vitamins separate out into the outer medium not only as a result of the exchange of lower (and higher) organisms but also in the decomposition of cells and tissues. According to Meysel (1950), many microorganisms adjusted themselves to the use of such fragments.

These factors, of course, influence the specificity of the microbiological methods, together with the well-known capacity of microbes to synthesize, under definite conditions, one or another "growth substance"

which they are called upon to determine. In addition, the requirement by a microbe for definite components of the feeding medium can vary, depending on their quantitative proportions or on the presence of other substances; this was established by Meinke and Holland (1948, 1949) in the case of the aminoacids serine and threonine and by the same authors (1950) in the case of calcium ions. Finally, the growth of the microbe can be influenced by various, as yet incompletely investigated, substances, the action of which can retard or activate this growth (Iyerusalimskiy, 1947; see also Emery and other, 1950).

In a paper by Mardashev (1950), the outlook for the use of microbiological methods for biological materials (proteins), without prior hydrolysis, is rather alluring.

The problem of the possible application of microbiological determinations for such biological materials, such as animal and plant tissues, blood plasma, urine, etc, is of special interest. One cannot disagree with Mardashev (1950) who warned against the uncritical attitude, widespread among American investigators, regarding the use of microbiological methods for such purposes. It is sufficient to note the results of the investigations by Williams and Wieger (1945), later confirmed indirectly by Axelrod and others (1948) and by Treger (1948), which point out the stimulating influence of fats and fatty acids on the growth of microbes (used for the determination of biotin). This problem, however, requires further examination, since there are contradictions in the instructions of the American authors. As an illustration, we wish to point out the data by Tomarelli and others (1950) regarding the retarding influence of fatty acids on the growth of certain lactobacilli. Meysel (1950) shows that it is possible, in some instances, to replace para-aminobenzoic acid with purines and aminoacids (methionine). It is sufficient to cite the data by Hodson (1949) regarding the possible replacement of folic acid with desoxyribonucleoinic acid for some indicator microorganisms and, finally the data by Light and Clarke (1943) and Stokes and Martin (1943) regarding the influence of different concentrations of glucose on the growth of indicator microorganisms, in order to become convinced of the existence of various possible sources of errors in the application of microbiological methods for determining the above named (and other) substances in biological material. Therefore, the methods of preparing biological liquids and tissues for analysis with aid of microorganisms require extra care for the elimination of the interfering influence of these substances.

Microbiologists are well aware of the fact that the growth reaction does not always reflect completely the requirement by the microbe culture for one or another vitamin (see Iyerusalimskiy and Nerorova, 1946; Meysel, 1950).

The specificity of the microbiological methods appear incomplete because the vitamins can be present in the biological material in the form of compounds with proteins or other substances and, in such a form, cannot influence the growth of the microbes. According to Burkholder and others (1945)*, more than half of the vitamin in some food products is found in a

combined form. In such instances, in order to make the test substance available for the action of microbes, it is first necessary to liberate it by preliminary hydrolysis (including enzymatic hydrolysis).

*The presence of combined ascorbic acid in tissues was conclusively shown by the investigations of Gol'dsheyeyn and others (1947, 1950a 1950b), Mashbits (1949), and Sumtsova (1950).

The fact of stereochemcial "discrimination" by microorganisms is also a well known fact. For example, ordinarily, microbes use only l-aminoacids. Yet according to Steel and others (1949), there are also such cultures of microbes which do not differentiate between the d- and l-forms of aminoacids. Although the latter case is still only one example of the non-specificity of microbe action, still, Mardashev (1950) points out correctly that, in microbiological determinations, this difficulty can be avoided by using two such cultures of microbes, of which one is strictly specific for the determination of a definite optical antipode (levorotatory form), while the other uses both forms (levo- and dextrorotatory). In this manner, it is possible to determine the content of both optical antipodes (Dunn and others, 1947).

In order to familiarize the reader with the different stages of a microbiological method, we will describe, as an example, the well known method for the determination of glycocoll (glycine) in proteins. Three ml. of 10 percent HCl is added to 30-40 mg. of protein in a 15-ml. ampoule, the ampoule is sealed, sealed, and hydrolysis is carried out for 5 hrs. at 110°C. After cooling, the hydrolsate is transferred quantitatively into a 25-ml volumetric flask, water is added to the mark, and the flask is kept (under a layer of toluene) in a refrigerator. In order to make a determination, an aliquot portion of the hydrolysate is tranferred into another 25-ml. volumetric flask; at first, 5 N caustic soda is added and then 0.05 N caustic soda until a pH of 7.8 is reached, after which, water is added to the mark.

First, a complete synthetic medium (not excluding glycine) is prepared in accordance with the table, in order to adapt the culture of microbes to this medium. The determination is carried with the aid of Leuconostoc mesenteroides P-60, the daily agar culture of which is adapted with 24 hours to the synthetic medium of the same composition as that used (later) in the experiment. After a day, the culture of bacteria is centrifuged for 20 minutes at 5000 r.p.m., the liquid is decanted, and the residue is shaken in physiological solution. The turbidity of the bacterial suspension is set at a constant value with the aid of a nephelometer, after which, a control bacterioscopy is carried out.

In order to construct a standard curve, 5 ml. of glycine-free synthetic medium are measured into each of ten test tubes. A standard solution is prepared consisting of 2 mg. percent (accurately!) of glycine which had been previously dried in vacuum at 30° and kept in a vacuum desiccator with calcium chloride. Continuously increading amounts (0.5 to 5 ml.) of this standard solution are measured into each of the

ten test tubes containing the synthetic medium. The final volume of the contents in each test tube is diluted with water to 10 ml. and all the test tubes are left in an autoclave for 30 minutes under a pressure of 1 atmosphere, after which, each test tube is inoculated with the daily culture of Leuconostoc mesenteroides P-60 (previously adapted to the synthetic medium) and incubated in a thermostat for 72 hours at 37°

After this time interval, the intensity of the growth reaction of the microbe is determined by titrating the lactic acid in each test tube with 0.05 N caustic soda.

On the basis of the titration results, a standard curve is constructed showing the amount of lactic acid formed as a function of the concentration of the determinable glycine.

At the same time, a quantatative determination is made of the glycine in the test albumin. For this purpose, different amounts (in ml.) of the diluted albumin hydrolysate are measured into test tubes containing a synthetic medium of the same preparation and water is added to a volume of 10 ml; this is followed by inoculation of the culture, incubation in a thermostat, and titration with 0.05 N caustic soda (as prescribed for standard solutions). The glycine content is calculated from the standard curve (taking into account the degree of dilution of albumin hydrolysate).

On the basis of the above, it is possible to state that practically every microbiological method consists of the following stages: (1) preparation of laboratory ware and apparatus, (2) preparation of the media and the culture of a definite microbe, (3) preparation of standard solutions of the test sample, (4) sterilization (autoclaving), (5) performance of actual experiment, (6) inoculation of the culture and incubation in a thermostat, and (7) recording the measurements, in particular, measurements of the intensity of growth of the microbe (turbidity, acidity, weight).

Preparation of Laboratory Ware

The need of thoroughly washed and sterilized laboratory ware for microbiological determinations need hardly be demonstrated. This can be most simply accomplished by washing the test tubes with water and then immersing in a bichromate solution (1000 gm of concentrated sulfuric acid is carefully added, while stirring, to 100gm of hot 50 percent sodium bichromate solution), leaving overnight in a steam bath, draining the liquid, removing traces of the liquid by repeated washing with tap water and then with distilled water, immersing the test tubes in a glass vessel with distilled water, covering the vessel, and autoclaving for 20 minutes.

Preparation of Media

The success of the microbiological method depends greatly on the thorough preparation of the synthetic media which must contain all the aminoacids,

vitamins, purine and pyrimidine derivatives, and also carbohydrates, mineral salts, and water necessary for the normal growth of the microbe. As is well known, the principle of the microbiological method is based on the exclusion from the synthetic medium, in which the microbes grow, of that component which is determined quantitatively from the intensity of growth of the microbes. The latter should be proportional to the concentration of the given component (this must first be determined thoroughly).

The substances necessary for the normal growth, development, and bacterial multiplication, in very small amounts besides, are customarily called growth substances or factors of growth. This term is usually applied to vitamins, specific aminoacids, and purine and pyrimidine derivatives. However, as it correctly pointed out by Meysel (1950), there are no special growth substances. The growth of cells and of the organism is the summary result of the most diverse processes, th disturbance of which can be brought about by the exclusion of any link from their chain and not just o vitamins or irreplaceable aminoacids. Thus, by making a quantitative determination of any component of the synthetic medium, we are pursuing strictly practical analytical ends, without predetermining the physiological significance of the given component.

We wish to note, however, that th microbiological methods have undoubtedly exceeded their importance as purely technical procedures, since the work of preparing the synthetic media, in connection with the development of these methods, leads to an accumulation of facts which are instrumental in revealing the nature of the exchange processes of the substances.

It follows from the above that diverse and , besides, specially pure reagents, are necessary for the preparation of synthetic media. As an example, we will cite the composition of media employed in different methods for determining aminoacids (Dunn, 1949).

The given table is a graphic example of the complex composition of synthetic media which contain up to 50 different components.

Within recent years, Anglo-American investigators have proposed various modifications of the methods; these have frequently been dictated only by possession of special reagents and by desire to suggest their "own" modifications. In particular, the method of the American investigators Cardiral and Hedrick (1948), as shown in the table, inspires little confidence. The basis of their proposed modification is the work by Shankman (1943); however, this paper has no data that folic acid is also a constituent of the synthetic medium. On the other hand, Cardiral and Hedrick (1948) indicate that they increased tenfold the concentration of the folic acid.

In the light of the above, the fruitful efforts of Soviet biochemists toward selecting means for simplifying the microbiological methods are of special interest. Some Soviet authors utilized pepton treated with

hydrogen peroxide as a source of aminoacids. As a result of such treatment, it is necessary to add to the medium only tryptophane, tyrosine, methionine, and cystine, since all the remaining aminoacids are thereby retained. The authors have used with success Russian-produced pepton for an equivalent medium (in determining tryptophane, this aminoacid is, of course, excluded from the medium).

Formulations for media (see, for example, Snell, 1948) used in microbiological methods for the determination of vitamins are ordinarily also quite complex and require scarce reagents, even when these media are semisynthetic and prepared, for example, with casein hydrolysate separated from vitamins.

In this respect, the microbiological method of determining folic acid, as proposed by Pershin and Shcherbakova (1951), is of interest. These authors were successful in preparing a simple medium having as its base salts, glucose, and fermentation products of casein. Folic acid was removed from this by repeated adsorption with charcoal at a pH of 3; in this process, nicotinic and parthotenci acids are lost and must be added to the medium, whereas, the addition of other vitamins is not necessary. Persov and Scherbakova prepared several such media which gave analogous results in the determination of folic acid in synthetic preparations. The authors point out correctly that the use of this method for the determination of folic acid in biological materials requires special study.

The compositions of some synthetic media proposed most recently for microbiological methods are given in a paper by the author (1951).

As pointed out above, media of comparatively simple composition are used for the determination of folic acid with the aid of neurospora or yeast fungi. Recently, Odintsova, Meysel, and Guseva (1951) have made a critical evaluation of the methods proposed by foreign authors for the determination of vitamin B_1 by utilizing its cocarboxylase action during the cleavage of pyroracemic acid or of the fermentative reaction of the microorganisms on vitamin B_1 (see also Odintsova, 1949). These Soviet investigators have proposed a method of determining vitamin B1, vitamine thiazole, and cocarboxylase by utilizing the fermentative method with the culture Endomyces magnusii in a sugar-phosphate medium of rather simple composition. The starting material can be any commercial sugar powder, treated with activated charcoal and added to the agarmineral medium, to which 0.1 percent yeast extract is also added as a source of vitamin B1 and biotin. The sensitivity of this method was noted by Smirnov and Dykman (1949) who used it for the determination of the content of thiamine in the nervous system of carp fish.

Further investigations by Soviet scientists will undoubtedly lead to a still further simplification of the composition of synthetic media; this will be a considerable stimulus for the wide adaptation of microbiological methods in biochemical investigations.

Microbiological methods, which permit the use of smaller amounts of the synthetic medium and of reagents, in general, have been under development

TABLE 1

COMPOSITION OF BASIC MEDIA OF LACTO BACILLI USED IN THE DETERMINATION OF AMINOACIDS BY DIFFERENT AUTHORS
(Compiled by Dipp, 1948)

Components	Orel and Fraton	Patton and others	Cardinal and others		Velik and Rontoni			Meiahe and Holland				Heller and Karch
[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]
Glucose, gm.	2	1	1	2	4	1	2	2	1	1	1	2
$(NH_4)_2SO_4$, gm.	0.3			0.6		0.3			0.3			
Sodium acetate, gm.	2.0	0.6	0.6	1.2	2.4	0.6	1.2	0.6	0.6	0.6	0.6	1.2
NH_4Cl, gm.					1.2		0.6	0.3				0.6
$K_2H_2PO_4$, gm.	0.25	0.05	0.08	0.05	0.1	0.1	0.1	0.1	0.05	0.1	0.05	0.05
K_2HPO_4, gm	0.25	0.05	0.05	0.05	0.1	0.1	0.1	0.1	0.05	0.1	0.05	0.05
$MgSO_4 \cdot 7H_2O$, gm.	0.08	0.02	0.02	0.02	0.04	0.02	0.02	0.02	0.02	0.02	0.02	0.02
NaCl, gm.	0.004	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001
$FeSO_4 \cdot 7H_2O$, gm.	0.004	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001
$MnSO_4$ $4H_2O$, gm.	0 018	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0 001	0.001
Inositol γ						250		250		250		
Choline Chloride γ						250		250		250		
Thiamine hydrochloride γ	100	20		100	200	10	100	50	100	10	20	100
Pyrodixine hydrochloride γ	100	40	20	160	320	30	160	160	20	30	200	160
Ca panthotenate γ	100	20	20	200	400	10	200	20	20	10	20	200
Para-aminobenzoic acid γ	8	4		0.01	0.02	30	0.1	0.1	20	30	4	0 01
Riboflavin γ	100	20	20	200	400	10	20	20	20	10	20	200
Nicotinic acid γ	100	20	20	200	400	10	200	20	40	0.02	20	200
Biotin γ	0.5	0.02	0.01	0.5	1.0	0.02	0.6	0.1	0.5	10	0.02	0.5
Folic acid γ	0.5	0.2	10	200	0.4	1	0.4	0.4	0	1	0.2	0.2
Adenine sulfate, mg.	1.0	1.0	2.0	1.2	2.4	1.0	1.2	1.0	5	1	1.0	1.2
Guanine hydrochloride, mg.	1.0	1.0		1.2	2.4	1.0	1.2	1.0	5	1	1.0	1.2
Uracil, mg.	1.0	1.0		1.2	2.4	1.0	1.2	1.0	5	1	1.0	1.2
Xanthine, mg.	1.0					1.0						
Alanine, mg.	20	20	20	200	400	200	100	20	40	20	20	100
Asparaginic acid, mg.		20		+	+	40	200	40	40	40	20	
Asparagine, mg.	40		20	40	80							40
Arginine hydrochloride mg.	10	20	20	8	16	10	20	10	20	10	20	40
Cystine, mg.	10	20	20	12	24	10	20	10	20	10	20	12
Glutamic acid, mg.	50	20	20	15	30	100	80	40	40	100	20	15
Glycine, mg.	10	20	20	10	20	10	10	10	20	100	20	10
Histidine hydrochloride mg.	10	20	20	10	4	10	10	10	20	10	20	2
Hydroxyproline, mg.		20	20		20				20	10	20	
Isoleucine, mg.	20	20	20	15	30	20	20	20	20	10	20	15
Leucine, mg.	10	20	20	7.5	15	10	20	20	20	10	20	7.5
Lysine hydrochloride mg.	20	10	20	16	32	10	20	10	20	10	10	16
Methionine, mg.	20	20	20	4	8	20	10	20	20	20	20	4
Norleucine, mg.		20	20	10	20	20		20	20	20	20	
Norvaline, mg.				10	20	20				20		
Phenylalanine, mg.	20	20	20	8	12	20	10	20	20	20	20	8
Proline, mg.	10	20	20	2.5	5		10	10	20	10	20	2.5
Serine, mg.	10	20	20	8	16	20	+	+	20	20	+	8
Threonine, mg.	20	20	20	45	90	20	+	+	20	20	+	45
Tryptophane, mg.	10	40	20	1.0	2	10	5	10	20	10	40	1.0
Tyrosine, mg.	10	20	20	3.0	6	10	10	10	20	10	20	3.0
Valine, mg.	20	20	20	15	30	20	20	20	20	20	20	15

within recent times. A description of such "micromethods" is given in our paper (Asatiani, 1951).

Preparation of the Sample and of the Standard Solutions

The preparation of the material for analysis is dependent on the type of compound subject to quantitative analysis.

In preparing the material by the method of enzymatic hydrolysis, it is necessary to take into account the content of l-tryptophane in the enzyme preparations. For this purpose, a simultaneous control experiment is carried out under identical conditions by subjecting the enzyme preparations to hydrolysis. The content of l-tryptophane in the protein hydrolysate is calculated by subtracting from the content of l-tryptophane determined in the main experiment the content of this aminoacid in the enzyme preparation.*

* Rabinwitch and other (1948), considering that the enzyme hydrolysate of casin can contain substances which will affect the growth reaction of the microbes, recommended that these be removed by twofold treatment of the hydrolysate with charcoal. See also Pershin and Shcherbakova (1951).

Although the enzymatic hydrolysis is the natural and least harmful method of preparing protein for the determination of its aminoacid composition, still, as Mardashev (1950) points out, the products of such hydrolysis can contain peptides and the action of these on the microbes can alter the results of the determination. This is confirmed by Dunn and McClure (1950) who studied the growth reaction of lactobacilli in the products of the partial hydrolysis of proteins and also by Klungsoyr and others (1951).

The preparation of tissues and blood for the determination of free aminoacids and of the total amount of aminoacid by the microbiological method is described below.

The animal is killed and the tissue is immediately removed, wrapped in filter paper, and then placed in a thermos bottle containing dry ice (solid carbon dioxide). The frozen tissue is weighed, triturated with a cooled pestle in a mortar (into which pieces of dry ice are thrown) to the consistency of coarse sand, and transferred, together with the dry ice, into a glass which is heated on a water bath to remove the greater portion of the carbon dioxide.

The proper amount of 0.013 N acetic acid (10 ml. per gm. of tissue of liver, kidney, and spleen and 5 ml. per gm. of heart or skeletal muscle) is added to the glass and the mixture is rapidly heated and boiled for 4 minutes with constant stirring.

Then follows homogenization for 10 minutes in a Waring blender, after which the suspension is again boiled for 4 minutes, then it is diluted to the proper volume and filtered.

One volume of 0.6 N sulfuric acid is added to ten volumes of the filtrate and a sufficient amount of 10 percent sodium tungstate solution for the complete precipitation of the proteins. Ordinarily, 1 ml. of this solution is sufficient for an amount of tissue extract corresponding to the following amounts of tissue samples: skeletal muscle 7.5 mg., liver 5 gm., kidney 2 gm., heart muscle 1gm., and spleen 0.8 gm. A small excess of tungstic acid does not interfere with the subsequent microbiological determination.

After a 15 minute rest, the solution is filtered and caustic soda solution is added to filtrate to give a pH of 7.

For microbiological determinations; solutions of different concentrations are prepared (60 mg. of muscle tissue, 25 mg. of liver tissue, and 15 mg. of kidney or spleen tissue per ml.). The samples are kept in a refrigerator under toluene prior to analysis.

In order to determine the total amount of non-protein free aminoacid of the test tissue*, 1ml. of concentrated hydrochloric acid is added to 20 ml. of the concentrated tungstate filtrate of the tissue and the mixture is then autoclaved for 4 hours at 120°. At the end of the autoclaving, the pH of the solution is adjusted at 7 and the solution is diluted to a definite concentration for the microbiological determination.**

*This includes the total content of the given aminoacid in the hydrolysate of the protein free filtrate, including also the "free" form of aminoacid which is subject to microbiological determination prior to hydrolysis.

**It is necessary to point out that, in autoclaving, partial decomposition (or racemization) of some aminoacids is always possible. The extent of the resulting error is ignored by American authors.

In order to determine the aminoacid composition of the entire tissue after hydrolysis, the exact sample (1-2 gm.) is placed in a glass and hydrolyzed by autoclaving with 20 ml. of 6 N hydrocloric acid for 10 hours at 120°. The sample is then adjusted at a pH of 7 and diluted to a concentration of about 0.5 mg of tissue per ml. The determination of individual aminoacids is made by the microbiological method.

The determination of free aminoacids and blood erythrocytes is carried out in the following manner. The volumes of plasma and erythrocytes in the heparinized blood is determined first, then the plasma is separated and a protein-free tungstate filtrate is prepared from it in the usual manner.

As regards the erythrocytes, the upper layer is first removed by suction from the residue of plasma and leukocytes, then 1 volume of the erythrocytes (sample of erythrocytes is weighed and the weight is multiplied by 1.092 to convert it to a volume basis) is hemolyzed by the addition of 4 volumes of water, after which it is allowed to stand for 20 minutes.

While shaking the hemolyzed mixture energetically, 1.54 volumes of 10 percent of sodium tungstate solution and 1.65 volumes of 0.667 N sulfuric acid are rapidly added. The precipitate is filtered off and the transparent filtrate (which should be free of tungstate and proteins) is neutralized and thickened by boiling down to half its volume. The plasma and the erythrocytes (filtrates) are kept frozen under toluene prior to analysis. Ordinarily, 1 ml. of the filtrate of erythrocytes corresponds to 0.25 ml. of erythrocytes and 1 ml. of the filtrate of the plasma to 0.33 ml. of plasma.

The determination of the individual aminoacids in these filtrates is accomplished with the aid of microbiological methods (Johnson and Bergeim, 1951).

A similar method for the preparation of tissues for microbiological determination fo free aminoacids is used also by Shurr and other (1950) who performed the analysis with a tungstate, protein-free sediment of tissue homogenate.

As regards urine, the microbiological determination of aminoacids therein is usually made by acid hydrolysis (Dunn and others, 1949; Camien and Dunn, 1950).

As regard vitamins, almost all the microbiological methods of their determination are utilized only after the given vitamin has been liberated from its "combined" form which ordinarily makes it unavailable to the action of the microorganism. It should be borne in mind that in different tissues the given vitamin can be present in differently combined forms and that, on the other hand, some microbes have the capacity to utilize also the combined form of the vitamin (Meyse], 1950).

It follows from this that the choice of the method of extracting the vitamins* depends on the type of microbe in the tissue to be analyzed.

*Description of Microbiological Methods of Determining Vitamins (Asatiani, 1949, 1951)

The following methods of preparing material for the determination of vitamins are the most widely used (Snell, 1948).

For the determination of biotin, hydrolysis by autoclaving at 120°C and with 0.5 N sulfuric acid for 1-2 hours are usually employed. More prolonged heating can result in partial decomposition of the biotin.

For the determination of choline, the material is prepared by autoclaving with 10 ml. of 3 N sulfuric or hydrochloric acid per 100mg. of dry, triturated material.

For the determination of folic acid, the sample is thoroughly triturated with water, cooled, and subjected to fermentative hydrolysis at a pH of 4.5 for 16 hours at 45°. The ferment preparation is obtained from the kidneys of pigs or from the pancreas of chicks. For the determination

of inosite, the sample is hydrolyzed by boiling with a reflux condenser in the presence of an excess of 18 percent hydrochloric acid for a period of 6 hours, after which the hydrochloric acid is distilled off in vacuum, while the residue is dissolved in water and adjusted at the proper pH.

For the determination of nicotinic acid, the thoroughly triturated sample is shaken with a sufficient amount of N sulfuric acid to give a concentration of 1 microgram. of nicotinic acid per ml. of mixture, which is autoclaved (pressure of 1 atm.) for 15 minutes, then cooled, and neutralized with caustic soda.

Panthotenic acid is determined after prior autoclaving of the sample, triturated with water to a concentration of about 6-20 microgram percent (pH of about 7), for 15 minutes (pressure of 1 atmosphere); after cooling, is first subjected to fermentative hydrolysis* and filtered. The determination is carried out with a transparent filtrate.

In order to determine riboflavin, the thoroughly triturated sample is shaken with a tenfold amount (by weight) of 0.1 N hydrochloric acid, autoclaved for 15-20 minutes (pressure of 1 atmosphere), cooled, pH adjusted at 4.5 by addition of 2.5 ml. of sodium acetate solution, and filtered (it is possible to make the determination also after fermentative hydrolysis.

For the determination of thiamine, the thoroughly triturated sample is shaken with a 15-fold amount (by weight) of 0.1 N sulfuric acid, heated for 30 minutes on a water bath (taking care not to allow the pH to rise above 1.5), cooled, and adjusted at a pH of 4-4.5 by the addition of sodium acetate solution; after this fermentative hydrolysis is carried out by adding 1 ml. of 10 percent takadiastase solution or klarase for every 10 micrograms of thiamine and incubation is accomplished in 3 hours at 45-50° or overnight (under toluene) at 37°.

The determination of vitamin B_6 is performed with the suspension of the sample containing 2 micrograms of vitamin in 180 ml. of 0.055 N hydrochloric acid. The mixture is autoclaved for 5 hours (pressure of 1 atmosphere) cooled, and adjusted at the proper pH.

*Instead of the ferment preparation "mylase P", recommended for this purpose, it is permissible to use the preparations takadiastase (10 percent solution), klarase, or other similar ferments (pH of about 4.5, incubation for 12-24 hours at 45-50°). In general, the preparation of material with the aid of fermentative hydrolysis, i.e., the most natural method, should be given preference in those instances in which there is certainty of conditions which assure complete hydrolysis and exclusion of substances that may exert an influence on the intensity of the growth of the microbe and, thereby, on the accuracy of the determination.

The preparation of standard solutions for the construction of a standard curve and the collection of experimental data for the curve constitute a critical factor, upon which the acceptability and accuracy of

the method depend. The whole experiment for the establishment of the responsive growth reaction of the microbe is carried out by adding different amounts of this compound (in the form of standard solutions of different concentrations) to the equivalent sythetic medium from which the test compound is excluded. The various standard solutions are treated the same as the test samples. The amounts (usually in micrograms) of the substance to be determined (aminoacid, vitamin) are plotted at the bottom, along the abscissa of a system of axes, while the amounts (in ml.) of alkali used in the titration are plotted from the left, along the ordinate Points are marked at the intersections of the lines extending upward (from the number denoting the content of the substance) and horizontally (from the number indicating the amount of alkaline solution used). A curve is then passed through these points, joining the points with straight lines. In a "good" standard curve, all the points lie on the same straight line.(within certain limits).

In a similar manner, a standard curve can also be constructed from the results of turbidometric or gravimetric analysis.

Unfortunately (since time and materials are required), the standard curve is usually not prepared "once and forever," but for each individual experiment, parallel with basic determination. The cause of this lies in the difficulties involved in keeping track of all the factors which can affect the results of the determination, i.e., the intensity of development of the microbe. In this respect, considerable interest was aroused by the attempts of our own investigators (Mardashev, 1950) to develop conditions (use of a medium of the same preparation and retained under sterile conditions) which would permit the use of the same standard curve for a whole series of experiments.

Sterilization

Autoclaving can, undoubtedly, bring about chemical changes in the composition of the medium. It is interesting to note that some lactobacilli grow poorly in non-autoclaved media. The cause of this phenomenon is still not clear, although, as pointed out by Snell (1948) in the case of Streptococcus faecalis, it is the reducing substances which can be replaced with cysteine. This problem was defined more accurately by Schweigert and others (1950) who showed that the growth reaction of Lactobacillus leichmannii in glutathoime is greater than in cystine or cysteine, if these substances are contained in the feeding medium prior to sterilization, and alike, if they are introduced into the feeding medium after sterilization. In other substances (Streptococcus salivarius), the growth of the microbe takes place after the addition of acetic aldehyde to the non-autoclaved medium. Usually, the carbohydrate component of the medium decomposes during the autoclaving, especially in the case of a neutral or alkaline reaction and in the the presence of phosphates and oxygen. Autoclaving at a high temperature can produce a reaction of the glucose with the aminoacids in the medium. As regards cystine, such a reaction (accompanied by destruction of the cystine) was observed evenduring moderate autoclaving.

Pyridoxine, puridoxal, thiamine, and vitamin B12 can undergo changes during autoclaving (Hendlin and Soars, 1951). Finally, a shortcoming of autoclavi is also the non-unformity of the results, which is caused by

the unlike distribution of the material in the autoclave. In order to avoid (even partially) the destructive influence of autoclaving, some investigators (Camien and Dunn, 1950, and others) have already after sterilization, added a sterile solution of glucose, under asceptic conditions, to each test tube.

In some instances, it is advantageous to replace glucose with saccharose (Patton and Hill, 1948). However, this is not always possible, inasmuch as certain types of lacto bacteria do not use saccharose (Camien and others (1947).

The interfering influence of autoclaving can, to certain extent, be compensated by the circumstance that both the sample and the standard solutions are subjected to autoclaving at the same time. In some, though rare, cases, when a medium with a low original pH and of relatively simple composition is used and the incubation period is not prolonged autoclaving can be replaced with sterilization by means of simple steaming (100° for 5 minutes). Complete elimination of the process of hot sterilization and autoclaving (if this were possible) would be the most radical solution of the problem.

In this respect, the statement by Mardashev (1950) that autoclaving can be replaced with cold sterilization through proper filters is of considerable interest. Further study of the problem will, obviously, show determinations. This would have great practical significance.

A general condition which would facilitate the use of microbiological methods is the possible complete standardization of all the conditions of the experiment. This being attained by using ware of the same quality and of the same dimensions and by operating always under the same conditions. In particular, it is necessary to create, as far as possible, the same conditions, for all the test tubes (these should be of the same size) in the autoclave and thermostat, not to shake individual test tubes selectively, either not to shake at all or to shake continuously all the test tubes. If a constant-temperature water bath, with constantly stirred water, is used instead of a thermostat, it is necessary to avoid uneven vibration of the test tubes with the inoculated medium; uneven vibration may result in different intensities of growth.

Inculation and Incubation

In a special paper dealing with the factor which influence the accuracy of microbiological methods, Toennies and Gallant (1948) state that the size of the drop of the bacterial suspension used for inoculation does not affect the results of the analysis. Experience on the part of Soviet investigators (Mardashev, 1950) indicate that these conclusions are groundless.

The number of microbe cells used for inoculation can, in most cases, affect the outcome of the determination. It is best to maintain the same dilution of the culture and the same size of drops of the bacterial suspension (with the aid of calibrated pipet or platinum ovelet) which is used

for inoculation, after the optimum concentration of microbes for each method has first been established, of course. In those instances, when this is possible, large dilution of the bacterial suspension makes it possible to use large volumes (1 ml.) for inoculation and, consequently, to make more accurate measurements.

The standardization of the inoculation conditions is accomplished more easily, if the basic medium is sterilized separtely before it is placed in the test tubes for the experiment. The medium is then cooled, inoculated, and the inoculatedmedim is distributed, under asceptic conditions, in prepared sterile test tubes for the experiment. Such a procedure was used successfull with yeast fungi (Snell, 1948).

The incubation conditions should also be constant and uniform for all the test tubes in which the determination is to be made by the same method. For this purpose, it is necessary to have a good thermostat or a thermostat room; the temperature variation should in no case exceed 1° (it is desirable that variations be much less). Snell (1948) recommends for incubation the use of water baths with a device for stirring the water uniformly.

In some instances, the lighting can affect the results of the determination (in particular, riboflavin and vitamin B_6 are sensitive to light). This should be taken into account, in order to provide the proper lighting (or to use ware of special glass).

Effecting the Measurements

In view of the considerable laboriousness of the microbiological methods, it is convenient to utilize devices which facilitate mass work. Such devices, including measuring instruments and others, are described in the book by Asatiani (1951).

As stated above, the most widely used method of measurement in microbiological methods is titration of the lactic acid with caustic soda solution of definite normality. In working with lacto bacteria (and with certain others), the amount of acid formed is ordinarily of the order of 10 ml. of 0.1 N acid per 10 ml. of the medium containing 1 percent glucose. This amount can be increased to 15-20 ml. of 0.1 N acid by doubling the amount of glucose in the medium (it is necessary to maintain the buffer effect of the medium at a sufficiently high level). Such a high output of lactic acid is all the more favorable, since it happens rarely. With proper skill, the measurement of the intensity of electrometric titration can increase the accuracy of the determination.

The turbidometric methods are used less widely, but they can give results of the same degree of accuracy as the acidimetric methods. It is only necessary that the test samples be optically "pure" and the turbidity during incubation should depend only on the growth of the microbe and not on any other factors. In order to obtain a uniform suspension, the test tube is shaken prior to photometering, after which one or two seconds are allowed for the air bubbles, which may form during

the shaking, to disappear from the suspension. Readings are made with a photoelectric colorimeter (nephelometer). A drawback in turbidimetry is the extremely dark coloration of the test sample; this is encountered rarely. With sufficient skill, the measurement of the intensity of turbidity can be accomplished much faster than titration with solution of alkali.

The turbidometric determination is used in methods which employ yeast fungi; the volume of the CO_2 formed is rarely measured in this case. The intensity of the turbidity is measured also in working with different (non-lactic) bacteria. In these instances, especially in working with Neurospora, the method of separating the outgrown mycelium (which is extracted with a needle or by filtration), with subsequent washing, drying, and weighing, is also sometimes employed. This procedure is, of course, more complex than titration or turbidimetry.

Lately, microbiological methods are being combined with other methods in biochemical investigations. As an example, we wish to point out the microbiological method of determining vitamin B_{12} in accordance with Winsten and Eigen (1950). The so-called method of "bioautographs," as developed by the authors, is based on the principle of the combination of chromatographic analysis with the microbiological method of determining vitamins.

The essence of the method of "bioautographs" is as follows. A drop of the solution containing the growth factors to be determined is investigated by the method of paper chromatography. The paper chromatogram is placed on the surface of the agar feeding medium which has been inoculated with the proper bacterial culture (the authors used Lactobacillus leichmannii 313.) The agar medium should contain all the factors necessary for the growth of the test microbe, with the exception of the test component.

After the removal of the paper strips and incubation in a thermostat at 37°, zones of the growth of the microve are formed in different sections of the location of the paper chromatograms. The position of the different growth factors under these conditions depends on the value of their Rf for the system of solvents* which was used for the development of the chromatogram. From this position, it is possible to characterize and identify the different factors present in the test sample.

*Rf is the coefficient of the rate of movement of the separable substances on the paper chromatogram.

At present, Soviet investigators are successfully using in biochemical investigations bacterial decarboxylases (Mardashev, 1949) in combination with chromatographic methods (Mardashev and Semina, 1949). There is less information available regarding the use of microbiological methods in combination with the method of tracer atoms. The application of this in microbiology is described in the review paper by Konikova and Kritsman (1951).

The usual accuracy of microbiological methods is ± 10 percent.

However, by observing all the conditions thoroughly, it is possible to improve this accuracy considerably.

The range of application of microbiological methods in biochemical investigations is continuously expanding. It is sufficient to point out that, in addition to aminoacids and water-soluble vitamins, microbiological methods are used for the determination of pyrimidine bases (Merrifield and Dunn, 1950), antibiotics (Sager and Arrigoni, 1950), fat-soluble vitamins D_2 and D_3 (Kodicek, 1950), sulfur (McManus and others, 1950), and various other substances.

As already pointed out above, the suitability of a microbiological method is determined by the following means:

(1) agreement between results of several determinations of the test substance (added to the test sample);

(2) agreement between results of the determination of the same substance with the aid of microorganisms;

(3) agreement between results of the determination by microbiological and other methods (chemical etc);

(4) agreement (after proper conversion) between results of the determination of different concentrations of the same substance.

(5) stability (reproducibility) of results with the same method.

This summary gives the basic criteria of the suitability of microbiological methods, which, while constantly being improved by Soviet investigators, will undoubtedly find the widest application in biochemical investigations.

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